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The balancing mucosal immune system

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General discussion

Mucosal immunity

The body is continuously in contact with both harmful pathogens and harmless commensals. Most of these interactions occur at the mucosal tissues such as the gastro-intestinal tract which facilitate the exchange of factors and which are therefore in close contact with the environment. The mucosal immune system is specifically adapted to discriminate between these harmful and harmless antigens. Inflammatory responses are developed in response to pathogens, whereas these responses are actively prevented under homeostatic conditions upon encounter of harmless antigens. This tolerance to harmless antigens is necessary to prevent food allergy and to maintain the symbiotic relation with commensal bacteria. These bacteria produce useful products for the host, while living in a stable and nutrient rich environment. In addition, the mucosal immune system directly interacts with the intestinal flora via pattern recognition receptors like TLR, leading to signaling cascades that support intestinal homeostasis.

Mucosal T cell responses

CD4⁺ T helper cell and Treg activation

DCs play a key role in the intestinal immune balance. They are present at the epithelial barrier, where they sample the luminal content and strive to generate the proper immune response by inducing the right type of T cell response^{1,2}. To provoke the proper immune response, mucosal DCs are conditioned by several cytokines like TGF- β , IL-10 and thymic stromal lymphopoietin (TSLP) and other factors such as retinoic acid (RA), produced by the mucosal environment.

DCs can induce different CD4⁺ T cell responses, divided into inflammatory T helper (Th) responses and regulatory T cell (Treg) responses. Th1 cells are known to produce IL-2 and IFN γ and activate cell mediated immune responses. Th2 cells produce IL-4 and IL-5 and stimulate IgE responses to fight parasites. The third T helper subset, Th17, produces IL-17 and IL-21 and is directed towards extracellular fungi and bacteria. Interestingly, Th17 deve-

lopment is closely related to Treg development since development of both T cell types is under influence of TGF- β . The inflammatory cytokine IL-6 directs the switch to Th17 differentiation, whereas Tregs are induced in the absence of IL-6 and the presence of RA. Tregs produce TGF- β and IL-10 and are essential for suppression of inflammatory responses and the prevention of autoimmune diseases and inflammatory bowel disease. In addition, food antigen specific Tregs are induced after oral ingestion of antigens, thereby inducing tolerance to food antigens and preventing food allergy. The induction of Tregs seems to be the default response during intestinal homeostasis.

CD8⁺ T cell suppression

Effector CD8⁺ T cells are specialized in lysis of cells that express non-self, like viral, antigens in the context of MHC class I molecules. It has been shown that mucosal DCs cross-present food antigens in MHC class I to CD8⁺ T cells in the MLN and that this results in CD8⁺ T cell tolerance³. Since mucosal DCs strongly activate Tregs and because Treg can suppress CD8⁺ T cells responses^{4,6}, CD8⁺ T cell oral tolerance could potentially be mediated via Tregs. Previous studies indicated that Tregs indeed can suppress the activation of food antigen specific CD8⁺ T cells^{3,5,6}, but it was not clear whether this mechanism was solely responsible for the suppression of CD8⁺ T cell responses. The data presented in *chapter 2* show that there is an additional pathway, since mice lacking CD4⁺ T cells still develop a suppressed CD8⁺ T cell response upon oral antigen feed. This pathway is possibly mediated by mucosal DC, but also plasmacytoid DCs or liver sinusoidal endothelial cells (LSECs) may be involved^{7,8}. LSEC have previously been shown to present oral antigens to CD8⁺ T cells and to induce deletion of these cells. In *chapter 2* we show that CD8⁺ T cells are deleted upon oral antigen administration, whereas after nasal antigen administration CD8⁺ T cells exhibit an anergic phenotype. An important difference between nasal and oral administration is the specific release of oral administered antigens into the bloodstream ending up via the portal vein in the liver. A possible scenario is that after oral administration LSECs take up oral antigens, and cross-present them to CD8⁺ T cells, causing CD4⁺ T cell independent deletion of CD8⁺ T cells, whereas after nasal administration mucosal DCs induce an anergic state.

Intestinal homeostasis and colitis

To maintain intestinal homeostasis, DCs are conditioned by the intestinal environment to suppress inflammatory responses and induce tolerance to harmless antigens. Several factors are involved in conditioning of intestinal DCs, like IL-10, TGF- β , thymic stromal lymphopoietin (TSLP) and retinoic acid (RA). Especially RA is very important in intestinal homeostasis, since RA is not only involved in conditioning of DC, but is also produced by conditioned DCs and is the key factor in the induction of Tregs. However, upon infection inflammatory factors are produced, leading to an inflammatory environment and a switch from suppressive into inflammatory DC⁹. Subsequently, these inflammatory DC induce immune responses that will enable the elimination of the pathogens followed by reversal to the suppressive intestinal environment.

In patients suffering from inflammatory bowel disease (IBD) the intestinal homeostasis is impaired leading to immune responses to the intestinal flora and strong intestinal inflammation. Reduced numbers of Tregs and reduced activity of Tregs are implicated in the pathogenesis in both human IBD and several mouse models¹⁰⁻¹². At the same time the induction of Tregs may be strongly impaired as a consequence of the ongoing intestinal inflammation. In both ulcerative colitis (UC) and Crohn's disease (CD) patients a defect in oral tolerance was observed¹³. Interestingly, this defect in tolerance induction was also disturbed in first-degree relatives of CD patients, pointing to a genetic risk of reduced Treg function resulting in IBD¹⁴.

Other studies have shown that both IBD patients with active inflammation as well as up to 10% of first-degree relatives exhibit increased intestinal permeability^{15,16}. This increased intestinal permeability could form another risk factor for IBD, since enhanced translocation of intestinal flora can activate immune responses and result in inflammation. Interestingly, no problems in barrier function were detected in the IBD families with reduced oral tolerance¹⁴. In *chapter 5* we have investigated whether chronic intestinal inflammation caused by reduced barrier function leads to defects in oral tolerance. Interestingly, no problems in tolerance induction were detected during the

acute phase of intestinal inflammation. However, during chronic inflammation we observed a defect in tolerance induction at DTH level. We hypothesize that during the acute phase of the disease conditioned DCs are still present in the intestines, whereas during chronic inflammation conditioning of newly incoming DCs is impaired. Interestingly, the defect in tolerance induction was observed at the DTH levels, but not at the CD8⁺ T cell levels, which may be due to remaining tolerance induction by the LSEC in the liver. Since DTH responses are mediated by CD4⁺ T cells and are suppressed by induced Tregs^{17,18}, our data suggests that during chronic inflammation the induction of Treg is hampered. In conclusion, reduced barrier function results in inflammation and defects in oral tolerance induction.

Microbiota and the mucosal immune system

Microbiota recognition and signaling pathways

The intestines provide a stable temperature and a constant nutrient flow for the microbiota that live in symbiosis with the host. At the other side, the commensal microbiota inhibit the growth of pathogens and produce vitamin K and short-chain fatty acids^{19,20}. In addition, the interaction between host and microbiota results in the formation of intestinal structures, like PP, villi and crypts during development^{21,22}. Finally, interactions with commensals sustain barrier integrity and mucosal immune homeostasis. An important role in these interactions is played by pathogen recognition receptors (PRR). Triggering of PRRs like TLR by the commensal flora leads to signaling cascades that support intestinal homeostasis. Interruption of the signaling cascade of TLRs results in increased severity of colitis as shown in mice lacking the adaptor protein MyD88 which is involved in most of the TLR signaling cascades²³.

Especially the TLR-2 signaling cascade is involved in maintaining intestinal homeostasis. A mutation in CARD15, increasing the NF- κ B activation by the TLR-2 signaling cascade, is more prevalent in patients with Crohn's disease and is thought to be involved in the pathogenesis of the disease²⁴. In line with this observation, in a mouse model of colitis using chemical disruption

of the epithelial barrier, TLR-2 signaling has been shown crucial as enhanced recovery of experimental colitis is induced by the synthetic TLR-2 agonist PCSK. This enhanced recovery is due to the TLR-2 stimulation that effectively preserves tight-junction-associated barrier assembly against stress-induced damage through promotion of PI3K/ Akt-mediated cell survival via MyD88²⁵.

Microbiota used to sustain intestinal homeostasis

Anticipating on the important role of microbiota in intestinal homeostasis and health of the host, bacteria and yeast that are normally present in food products like fermented milk products have been investigated for their effect on the immune system of the host. Bacteria that have been shown to have a health benefit for the host, are called probiotics. Effects of probiotics commonly involve induction of suppressive cytokines or stimulation of IgA responses²⁶⁻²⁹, but one of the complications in probiotic research is the complexity of signals induced by whole microbiota.

In *chapter 3* we have shown that *Saccharomyces cerevisiae*, a yeast with known TLR-2 activity, did not result in increased recovery of DSS colitis, whereas the synthetic TLR-2 ligand PCSK is known to support barrier function and thereby increase recovery from DSS colitis²⁵. This is probably due to the fact that *S. cerevisiae* expresses not only TLR-2 ligands, but also dectin-1 ligands that lead to an inflammatory signaling cascade³⁰.

We did observe increased B cell follicle formation during DSS colitis because of oral yeast treatment. Since B cells express TLR-2, this increased B cell follicle formation could be a direct consequence of yeast mediated TLR-2 signaling on B cells. Alternatively, recognition of yeast by other cells could result in the production of B cell attracting chemokines³¹⁻³³. In conclusion, we did not detect beneficial effects of yeast as a potential probiotic.

Myeloid-derived suppressor cells in DSS colitis

The induction of colitis by the addition of DSS to the drinking water of mice, is based on the disruption of the epithelial barrier leading to an overwhelming inflammatory response to the intestinal microbiota and tissue damage. The immunological response is not limited to the intestines, as shown in *chapter 4*, but also involves splenomegaly and increased serum cytokine levels, espe-

cially G-CSF. The splenomegaly is caused by accumulation of CD11b⁺Gr-1⁺ cells which morphologically resemble myeloid-derived suppressor cells (MDSC). MDSC have been described to be generated in a number of conditions, including in tumor bearing mice and during infections ^{34,35}. Immature myeloid cells are present in the bone marrow and spleen of all mice and differentiate into mature myeloid cells under normal conditions ³⁶. During infection and septic shock, these cells increase in numbers and demonstrate immunosuppressive functions like suppression of T cell responses ^{34,36}. One study has reported increased numbers of MDSC in an experimental model of colitis and in IBD patients ³⁷. Although we detected a strong increase in MDSC like cells during chronic colitis in *chapter 4*, we also showed that DSS treated mice developed normal CD8⁺ T cell responses after immunization in *chapter 5*. Interestingly, the expansion and activation of myeloid-derived suppressor cells are regulated by different pathways. Factors that induce MDSC expansion comprise cyclooxygenase 2, prostaglandins, M-CSF, GM-CSF, IL-6 and vascular endothelial growth factor (VEGF). These factors mainly induce signaling cascades that are involved in cell survival, proliferation, differentiation and apoptosis and converge on Janus kinase protein family members and STAT3 ³⁸. Factors that induce MDSC activation are mainly produced by activated T cells and tumor stromal cells and comprise INF γ , TLR ligands, IL-4, IL-13 and TGF- β . These factors activate signaling cascades that involve STAT1, STAT6 and NF- κ B ³⁹. Our observation of accumulation of MDSCs in the spleen of DSS treated animals without suppressive function indicate that mice with chronic colitis produce factors that induce accumulation of MDSC but not factors involved in MDSC activation.

In conclusion, the CD11b⁺Gr-1⁺ cells accumulating in the spleen of DSS treated animals are immature myeloid cells that resemble MDSCs lacking the suppressive function of MDSC.

Concluding remarks

The intestinal mucosal immune system continuously prevents inflammatory responses to harmless bacteria and food proteins, while inducing immune responses to invading pathogens. Interestingly, food proteins are not ignored to prevent inflammation, but an active tolerogenic response is provoked.

In this thesis, we hypothesize that this mucosal immune balance is a very vigorous system. Our data indicate that multiple mechanisms stabilize the balancing immune system. Besides Treg induction by oral antigen administration there are also Treg independent mechanisms active in the suppression of CD8⁺ T cells (*chapter 2*). Additionally, we show that the suppressive functions of the mucosal immune system are not affected by acute inflammatory processes in the intestines (*chapter 5*), whereas chronic inflammation only partially affected oral tolerance induction. On the other hand, we show that when the mucosal homeostasis is disturbed via DSS mediated damage to the epithelial barrier, it is difficult to modulate the inflammation (*chapter 3*).

From this thesis it is also clear that mucosal immune responses affect the whole body. Oral antigen application suppresses the systemic immune response upon subsequent immunization (*chapter 2*). Additionally, intestinal inflammation leads to increased serum levels of several cytokines and splenomegaly due to an increase of CD11b⁺Gr-1⁺ cells (*chapter 4*).

To improve the possibilities of modulating the mucosal immune system in order to cure and prevent diseases like IBD and food allergy, further research is warranted to unravel the suppressive mechanisms of the mucosal immune system and to understand the exact role microbiota are playing in the mucosal immune balance.

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